ACTA MICROBIOLOGICA POLONICA
1985, Vol. 34, No. 3/4, 301

Production of Organic Acids by Bacteria Isolated from Soil, Rhizosphere and Mycorrhizosphere of Pine (*Pinus sylvestris* L.)

HENRYK RÓŻYCKI

Laboratory of Microbiology, Institute of Biology, Nicolaus Copernicus University, 87-100 Toruń, Gagarina 9, Poland

Received 15 April 1985

Abstract

The bacteria studied released into the medium ten to eleven orgaric acids. Soil organisms excreted mainly pyruvic and α-ketoglutaric acids, while strains from the root zone — gluconic acid and unidentified uronic acid (y_2). Mean indices of total production of the organic acids by bacteria were in the following order: rhizosphere < soil < mycorhizosphere. Bacteria from the root zone released into the medium very large amounts of pyruvic, gluconic, and uronic (y_2) acids — in some instances several times higher than bacterial dry mass.

Introduction

The organic acids content in soil is mainly of microbiol origin (Slankis, Runeckles and Krotkov, 1964; Stevenson, 1967; Gussin and Lynch, 1981). These compounds are of great importance not only as pH lowering agents but also as easily available carbon and energy sources for microorganisms (Kunc and Macura, 1966) and as dissolving agents of some minerals (carbonates, phosphates, aluminosilicates) and chelating agents of polyvalent metal cations, making them available for plants (Duff and Webley, 1959; Stevenson, 1967). Furthermore, organic acids (both aliphatic and aromatic) are in different degree toxic to plants (Gussin and Lynch, 1981; Jalal and Read, 1983).

Up to date little work has been done on the production of organic acids by soil microorganisms. Therefore studies on these important organic compounds produced by soil and root zone microorganisms were performed.

Experimental

Materials and Methods

Bacteria. Bacteria — fifty strains isolated from soil, rhizosphere and mycorrhizosphere of pine (*Pinus sylvestris* L.) were used in our study. Details concerning the methods of sampling and identification of the bacteria were described previously (Różycki and Strzelczyk, 1985 a). Most of the organisms studied were pleomorphic types identical or similar to the genus *Arthrobacter* (Buchanan and Gibbons, 1974).

Culturing of bacteria. Bacteria were grown for 7 days at 26°C in the following medium: glucose — 10.0 g, NH_4NO_3 — 0.4 g, K_2HPO_4 — 0.2 g, „B" medium (Lochhead and Chase, 1943) without carbon and nitrogen source — 100 ml, cysteine 5.0 mg, thiamin — 10 μg, biotin — 0.01 μg, pyridoxin — 20 μg, nicotinic acid amide — 10 μg, riboflavin — 20 μg, inositol — 5 mg, vitamin B_2 — 0.2 μg, distilled water — 900 ml; pH 7.0—7.2. Bacteria were removed from the medium by centrifugation at 15 000 r.p.m. for 20 min.

Identification and quantitative determination of α-keto acids. α-keto acids were estimated by the modified Cavallini's method according to Clotten and Clotten (1962). Into 200 ml Erlenmeyer flasks with post culture liquids (50 ml) in water bath with constant temperature 37°C, was added 5 ml of 1% 2,4-dinitrophenylhydrazine solution in 2.5 M H_2SO_4 followed by incubation for 30 min. From the reaction mixture 2,4-dinitrophenylhydrazones of α-keto acids were extracted three times with 10 ml of the mixture; chloroform — ethyl acetate (4:1 v/v). The combined extracts were shaken with 15 ml of 0.5 M Na_2CO_3 and the carbonate extracts was cooled in ice bath and acidified with 5 ml of 2.5 M H_2SO_4. 2,4-dinitrophenylhydrazones of α-keto acids were reextracted from the obtained acidic solution with the above mentioned mixture (chloroform — ethyl acetate), and the combined extracts were evaporated et room temperature to dryness under reduced pressure (water pump). The dry residue was dissolved in 0.5 ml of 1,4-dioxane. In the same manner were prepared standards of α-keto acids derivatives which were applied instead of the post culture liquids. Into the sterile medium pyruvic and α-ketoglutaric acids in amounts 25 μg/ml were added.

For the separation and identification of α-keto acids the samples and standards were subjected to high voltage electrophoresis using Whatman No. 3 filter paper (28.5 × 46 cm) in 0.05 M Na_2CO_3 for 210 min. at 25—30 V/cm. 2, 5, 10 or 20 μl of the sample were applied to the paper depending on the expected concentration of 2,4-dinitrophenylohydrazones of α-keto acids. α-Keto acids derivatives were identified on the electrophoregrams as yellow spots.

In the quantitative determinations spots of α-keto acids derivatives were eluted with 1 M Na_2CO_3. For intensification of the eluate colour, 3 M NaOH was added and extinction of the eluates was measured in the spectrophotometer Spekol ZV (C. Zeiss, Jena) at the wavelength of 435 nm (Clotten and Clotten, 1962). The values obtained were compared with standard curves prepared for pyruvic — and for α-ketogluteric acid separately.

Estimation of other non-volatile organic acids. Post-culture liquids were passed through two connected columns (2 × 5.5 cm) with ion exchange resins: a) upper — with Dowex 50 W X8 (H^+ form, 100—200 mesh/; b) lower — with Dowex 1 X8 (CO_3^{2-} form, 100—200 mesh). The organic acids were eluated with 40 ml of 1 M $(NH_4)_2CO_3$ from anion exchange resin column. The columns were regenerated by passing: a) through Dowex 50—75 ml of 1 M HCl and subsequently by double distilled water until the neutral reaction was reached; b) through Dowex 1 — succesively: 40 ml of 1 M NaOH, 100 ml of double distilled water, 40 ml of 1 M Na_2CO_3 and double distilled water intil the neutral reaction was reached.

The ammonium-carbonate eluate containing anions of the acids was evaporated to dryness in vacuo at 45°C, and the dry residue was dissolved in 0.4 ml of 10% isopropanol.

The separation and identification of the organic acids in such obtained samples was carried out by paper high voltage electrophoresis according to Wang, Cheng and Tung (1967) — with modifications concerning the filter paper (Whatman No. 3) and the developing reagent (aniline-xylose reagent).

In the post-culture liquids of some rhizosphere and mycorrhizosphere bacteria the presence of gluconic acid and unidentified uronic acid (y_2) was stated. The above mentioned sugar acids were determined quantitatively on the basis of spot's surface measurements (uronic acid [y_2] in the glucuronic acid equivalents).

Statistical evaluation of the results of organic acids estimation was performed by methods described in a previous paper (Różycki and Strzelczyk, 1985 b)

Results

The results of our studies on organic acids production by bacteria are presented in Fig. 1 and in Tables I—II. Almost all bacteria studied relased organic acids into the medium (with the exception of 3 strains among the 50 studied). In general in post-culture liquids of the bacteria 10 (or 11) of different organic acids were detected: pyruvic, α-ketoglutatir, oxalic, malonic, malic (or citric), lactic, succinic, and gluconic acids and also two unidentified acidic compounds: y_1 and y_2 — uronic acid. Among single strains from 0 to 8 different acids were determined.

α-Keto acids were produced by all the soil bacteria as opposed to the root zone organisms. The sugar acids: gluconic acid and unidentified uronic acid (y_2) were released only by bacteria derived from the root

Table I

Indices of production of organic acids by bacteria*

Organic acids:	Soil	Rhizosphere	Mycorrhizosphere
Pyruvic	1.33^{a}	1.00^{ab}	0.25^{b}
α-Ketoglutaric	1.27^{a}	0.60^{b}	0.70^{b}
Oxalic	0.13^{a}	0^{a}	0^{a}
Malonic	0.13^{a}	0^{a}	0^{a}
Malic and/or citric	0.47^{a}	0^{b}	0.30^{a}
Lactic	0.67^{a}	0.07^{b}	0^{b}
Succinic	0.20^{a}	0^{a}	0^{a}
y_3 (unidentified)	0.33^{a}	0.20^{a}	0.30^{a}
Gluconic	0^{a}	0.73^{b}	2.70^{c}
y_4 (uronic)	0.13^{a}	0.60^{a}	1.35^{b}
Number of strains studied	15	15	20
Mean indices of total production of the organic acids ± 95% confidence limits	$0.467^{ab} \pm 0.132$	$0.320^{a} \pm 0.151$	$0.560^{b} \pm 0.126$

* Values indicated in the same lines by the same letter do not differ statistically ($P \leq 0.05$)

zone, first of all from the mycorrhizosphere (Fig. 1, Table I). The other acids were excreted into the medium by not nemerous strains (Fig. 1). It appears from the mean indices of total production of organic acids that rhizosphere bacteria were least active, the soil isolates being intermediate and the mycorrhizosphere organisms the most active producers of these compounds (Table I).

Table II shows the results of quantitative determinations of α-keto acids and sugar acids. The mean concentration of pyruvic acid in the post--culture liquids of rhizosphere bacteria was larger from the means for soil and mycorrhizosphere. However between the above mentioned values the

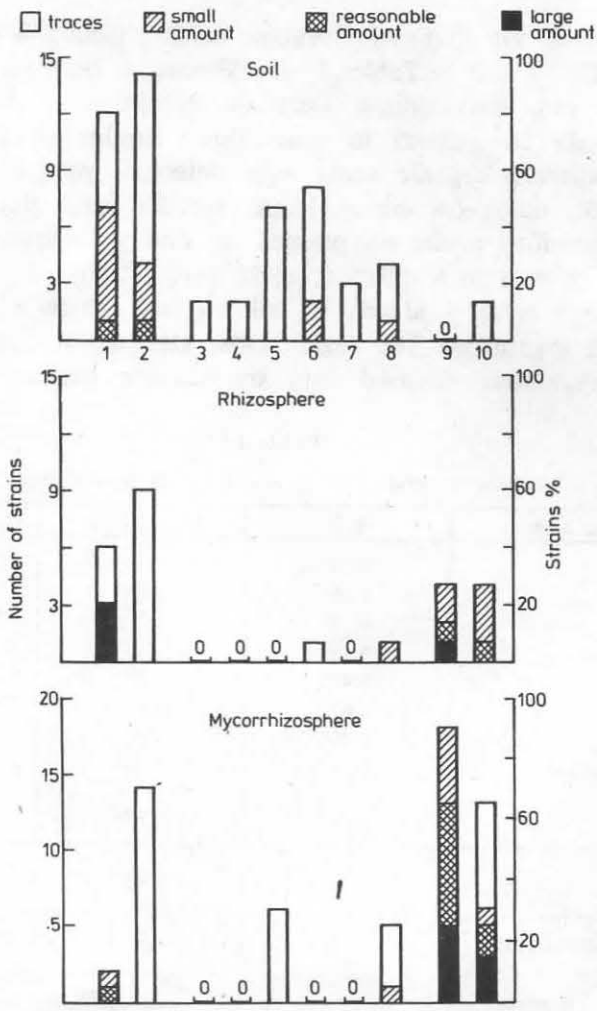

Fig. 1. Number and per cent of bacteria producing organic acids.

Explanations: Organic acids: 1 — pyruvic, 2 — α-ketoglutaric, 3 — oxalic, 4 — malonic, 5 — malic (citric), 6 — lactic, 7 — succinic, 8 — unidentified y_1, 9 — gluconic, 10 — unidentified y_2.

differences on significance level at $0.05 < P < 0.10$ were noted and in instance of the data calculated in relation to dry weight — $0.10 < P < 0.25$. The bacteria released less of α-ketoglutaric acid than pyruvic acid by one to three orders of a magnitude, at the same time soil strains released several times more of this compound than organisms from the root zone (Table II).

Table II

Amounts of organic acids produced by the bacteria[1]

Acids:	Source of isolation		
	Soil	Rhizosphere	Mycorrhizosphere
	α-Keto acids		
Pyruvic	[2] 3.46 ± 1.80	24.99 ± 9.65	3.99 ± 2.50
	[3] 27.55 ± 12.21	1032.20 ± 535.90	58.24 ± 40.81
α-Ketoglutaric	1.33 ± 0.37	0.08 ± 0.08	0.22 ± 0.09
	15.28 ± 6.24	0.72 ± 0.72	1.71 ± 0.80
Number of strains studied	10	4	5
Sugar acids			
Gluconic	[4] —	410.67 ± 160.52	584.89 ± 78.24
	—	3475.44 ± 1898.00	5399.19 ± 571.82
Uronic (y_4)	—	85.07 ± 49.67	129.00 ± 80.48
	—	906.44 ± 573.95	1052.97 ± 534.62
Number of strains studied	—	3	9

Explanations: [1] mean values ± standard error; [2] μg/ml of the medium; [3] μg/mg of dry mass; [4] not studied

Bacteria from the root zone produced in very large amounts sugar acids like gluconic and unidentified uronic acid (y_2). These organisms excreted several times more of gluconic acid than their dry weight. Amounts of unidentified uronic acid — y_2 were several times smaller than of gluconic acid (mean values for the rhizosphere and mycorrhizosphere were approximately the same as dry weight) — (Table II).

Discussion

The results of our studies show that bacteria of soil and the root zone of pine are capable of producing several organic acids. Soil bacteria released mainly α-keto acids, organisms derived from the root zone (especially from the mycorrhizosphere) excreted first of all sugar acids [gluconic — and uronic (y_2) acids]. Duff and Webley (1959) and Webley and Duff

(1965) have stated the ability of biosynthesis of an other sugar acid — 2-ketogluconic acid by bacteria of the genus *Pseudomonas* isolated from the root zone of agricultural plants. Chandrasekaran (1969) noted production of organic acids by microbial communities of soil and root zone of agricultural plants. These acids were rarely detected and only in trace amounts in post-culture liquids of the bacteria studied in this work (malic, citric, lactic, succinic, and oxalic acids).

The methods applied in this study did not allow the detection of the volatile organic acids (e.g. acetic, butyric and other acids). These compounds are as a rule produced during organic residues decomposition in anaerobic conditions or under limited oxygen pressure (Stevenson, 1967; Wang *et al.*, 1967; Gussin and Lynch, 1981). The bacteria studied in our work were grown in aerobic conditions and it is unexpected that they synthesized volatile fatty acids.

The majority of microorganisms studied in this work (with the exception of some bacteria producing sugar acids) produced α-keto acids — sometimes in large amounts. It is suggested that the release of pyruvic acid by bacteria is a result of unfavourable action of some environmental conditions as: inappropriate pH of the medium, limited oxygen pressure and others (Ruby and Nealson, 1977). The reason for pyruvic acid excretion by marine bacteria belonging to genera: *Beneckea, Photobacterium, Vibrio, Alcaligenes* and *Serratia* is probably the repressive action of glucose on the synthesis and activity of the enzymes acting in the Krebs cycle (Ruby and Nealson, 1977; Andreyeva, Vysotsky, Rodicheva and Scherbakova, 1981). Van Tigerstrom and Campbell (1966) found that *Pseudomonas aeruginosa* incubated in buffer enriched with glucose, but without nitrogen source excreted α-ketoglutaric and pyruvic acids. This process was inhibited by ammonium ions. Our results show that large amounts of pyruvic acid excreted by some rhizosphere bacteria were inversely proportional to growth intensity. It seems therefore that excretion of this compound may be a result of the metabolic block action. Amounts of pyruvic acid in the post-culture liquids studied in this work were similar to those recorded by Andreyeva *et al.*, (1981) using *Photobacterium mandapamensis*.

α-Keto acids are of importance not only as easily accessible carbon and energy sources for many soil microorganisms (Dahm, 1984). They are known to be important intermediate metabolites, from which amino acids are synthesized in the amination process.

Uronic acids produced by the mycorrhizosphere bacteria may be of importance as possible carbon sources for ectomycorrhizal fungi. Nylund (1981) observed that *Piloderma croceum* a fungus forming ectomycorrhizae with spruce (*Picea abies* (L.) Karst.] grew relatively good in the presence of galacturonic acid as the sole carbon source.

There is lack of quantitative data concerning sugar acids production

by bacteria. However it is known that 2-ketogluconic acid produced by rhizosphere pseudomonads dissolved intensively calcium phosphate and some aluminosilicates and also chelated polyvalent metal ions like: Ca^{2+}, Mg^{2+}, Fe^{3+} (Duff and Webley, 1959; Richards, 1974).

Decrease of the soil pH is caused among others by organic acids produced by microorganisms. Lower pH favours the development of fungi (Clark, 1967; Warcup, 1967). According to Pedersen (1970) and Lode and Pedersen (1970), fatty acids with 8—10 carbon atoms in the molecules acted toxically towards the ectomycorrhizal fungi. Further studies on the effect of environmental factors on the production of organic acids by microorganisms are required. Also the importance of these compounds in metabiotic relations needs further attention.

Acknowledgements. I express my best thanks to Prof. Dr. Edmund Strzelczyk for his suggestions during the course of the experiments and critical reading of the manuscript. This research was carried out under problem MR.II.16 coordinated by the Institute of Dendrology, Polish Academy of Sciences.

Literature

Andreyeva R. I., Vysotsky E. S., Rodicheva E. K., Sherbakova G. Ya., 1981: Production of pyruvic acid by the luminescent bacterium *Photobacterium mandapamensis* (In Russian with English summary). *Mikrobiologia*, **50**: 776.

Buchanan R. E., Gibbens N. F., 1974: Bergey's Manual of Determinative Bacteriology (VIII-th ed.). The Williams a. Wilkins Co., Baltimore.

Chandrasekaran S., 1969: Production of organic acids by soil microorganisms. *Plant. a. Soil* **30**, 299.

Clark F. E., 1967: Soil bacteria. Soil Biology. A. Burges and F. Raw (Eds.) Academic Press, London.

Clotten R., Clotten A., 1962: Hochspannungs-Electrophorese. Ihre Anwendungsmöglichkeiten für biochemische und klinisch-chemische Trennprobleme. Georg Thieme Verlag, Stuttgart.

Dahm H., 1984: Metabolic activity of bacteria isolated from soil, rhizosphere and mycorrhizosphere of pine (*Pinus silvestris* L.). *Acta Microbiol. Polon.*, **33:** 147.

Duff R. B., Webley D. M., 1959: 2-ketogluconic acid as a natural chelator produced by soil bacteria. *Chem. a. Industry*, **44**: 1376.

Gussin E. J., Lynch J. M., 1981: Microbial fermentation of grass residues to organic acids as a factor in the establishment of new grass swards. *New Phytol.*, **89**: 449.

Jalal M. A. F., Read D. J., 1983: The organic acid composition of Calluna heathland soil with special reference to phyto- and fungitoxicity. I. Isolation and identification of organic acids. *Plant a. Soil*, **70**: 257.

Kunc 3., Macura J., 1966: Decomposition of root exudates in soil. *Folia Microbiol.*, **11**: 239.

Lochhead A. G., Chase F. E., 1943: Qualitative studies of soil microorganisms. V. Nutritional requirements of the predominant bacterial flora. *Soil. Sci.*, **55**: 185.

Lode A., Pedersen T. A., 1970: Fatty acids induced leaking of organic compounds from *Boletus variegatus*. *Physiol. Plant.*, **23**: 715.

Nylund J. E., 1981: The formation of ectomycorrhiza in conifers: structural and physiological studies with special reference to mycobiont *Piloderma croceum* Erikss. and Hjortst. Doctoral thesis at Uppsala University.

Pedersen T. A., 1970: Effect of fatty acids and methyl octanoate on resting mycelium of Boletus variegatus. *Physiol. Plant.*, **23**: 654.

Richards B. N., 1974: Introduction to the Soil Ecosystem. Longman Group Ltd. Essex.

Różycki H., Strzelczyk E., 1985 a: Synthesis of free amino acids by bacteria isolated from soil, rhizosphere and mycorrhizosphere of pine (*Pinus sylvestris* L.). *Zbl. Mikrobiol.*, **140**: 41.

Różycki H., Strzelczyk E., 1985 b: Free amino acids production by actinomycetes isolated from soil, thizosphere and mycorrhizosphere of pine (*Pinus sylvestris* L.) *Zbl. Mikrobiol.* in print.

Ruby E. G., Nealson K. H., 1977: Pyruvate production and excretion by the luminous marine bacteria. *Appl. Environ. Microbiol.* **34**: 164.

Slankis V., Runeckles V.C., Krotkov G., 1964: Metabolites liberated by roots of white pine (*Pinus strobus* L.) seedlings. *Physiol. Plant.*, **17**: 301.

Stevenson F. J., 1967: Organic acids in soil. Soil Biochemistry, Vol. 1. A.D. McLaren and G. H. Peterson (Eds.) Marcel Dekker Inc., N. York.

van Tigerstrom M., Campbell J. J. R., 1966: The accumulation of α-ketoglutarate by suspensions of Pseudomonas aeruginosa. *Can. J. Microbiol.*, **12**: 1005.

Wang T. S. C., Cheng S. Y., Tung H., 1967: Extraction and analysis of soil organic acids. *Soil Sci.*, **103**: 360.

Warcup J. H., 1967: Fungi in the soil. Soil Biology. A. Burges and F. Raw (Eds.) Academic Press, London.

Webley D. M., Duff R. B., 1965: The incidence, in soils and other habitats of microorganisms producing 2-ketogluconic acid. *Plant a. Soil.* **22**: 307.